Short Communication

The unfolded protein response and programmed cell death are induced by expression of Garlic virus X p11 in *Nicotiana benthamiana*

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Garlic virus X (GarVX) ORF3 encodes a p11 protein, which contributes to virus cell-to-cell movement and forms granules on the endoplasmic reticulum (ER) in *Nicotiana benthamiana*. Expression of p11 either from a binary vector, PVX or TMV induced ER stress and the unfolded protein response (UPR), as demonstrated by an increase in transcription of the ER luminal binding protein (BiP) and bZIP60 genes. UPR-related programmed cell death (PCD) was elicited by PVX:p11 or TMV:p11 in systemic infected leaves. Examination of p11 mutants with deletions of two transmembrane domains (TM) revealed that both were required for generating granules and for inducing necrosis. TRV-based VIGS was used to investigate the correlation between bZIP60 expression and p11-induced UPR-related PCD. Less necrosis was observed on local and systemic leaves of bZIP60 knockdown plants when infected with PVXp11, suggesting that bZIP60 plays an important role in the UPR-related PCD response to p11 in *N. benthamiana*.

Abiotic and biotic stress can increase the load of unfolded and misfolded proteins that accumulate in the endoplasmic reticulum (ER), inducing a protective response termed the unfolded protein response (UPR), which is conserved across the animal and plant kingdoms (Koizumi et al., 2001). The role of the UPR is to orchestrate adaptation to ER stress and restore healthy ER function, prolonging cell viability. In contrast, programmed cell death (PCD) is triggered under severe or chronic ER stress, and acts to kill damaged cells thereby protecting other, unaffected cells (Sitia & Braakman, 2003). Viral invasion, with associated disruption of normal protein synthesis and cellular balance, can cause ER stress and the UPR in plants. It was previously shown that the Potato virus X (PVX) TGBp3, a viral movement protein, is an inducer of ER stress, which leads to increasing expression of bZIP60 and UPR-related genes such as ER luminal binding protein (BiP), protein disulfide isomerase (PDI), calreticulin (CRT) and calmodulin (CAM) (Ye et al., 2011). The P10 outer capsid protein of the

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Expression of GarVX p11 induces the UPR and PCD in *Nicotiana benthamiana*

**Fig. 1.** *Bip* and *bZIP60* transcription up-regulation following expression of GarVX p11 in *N. benthamiana* leaves. (a) qRT-PCR assay of *BiP* and *bZIP60* mRNA levels in leaves after transient expression of p11 from a binary plasmid (left panel) or from PVX (right panel). Mock is water-infiltrated, pCV : mRFP and PVX : GFP are control constructs. Total RNA was extracted using Trizol (Ambion). A PrimeScript RT reagent kit with gDNA Eraser (TAKARA) was used to prepare cDNA. Fast Start Universal SYBR Green Master (Roche) was used to perform qRT-PCR. All primer pairs are listed in Table S1 (available in the online Supplementary Material). Relative quantitation used the comparative C<sub>t</sub> method with an endogenous control gene (actin). Bars represent the standard errors of the means of three replicate assays. A two-sample unequal variance directional t-test was used to test significance of the difference (*, P<0.05; **, P<0.01). (b) Confocal image of epidermal cells expressing free mRFP (left panel) or p11-mRFP (right panel). Granules formed by p11-mRFP are indicated by white arrowheads. Bar, 25 µm. (c) PVX infection in infiltrated leaves at 3 dpi revealed by Western blotting of PVX CP. Lower panel shows Ponceau.
reovirus, Rice black-streak dwarf virus (RBSDV) localizes on the ER and induces the UPR during ER stress (Sun et al., 2013). There are two UPR pathways operating in plants. One is mediated by IRE1-bZIP60 and is thought to play an essential role in viral infection, whereas the other is mediated by site-1/site-2 proteases (S1P/S2P)-bZIP17/bZIP18), has a similar function to the animal ATF6 pathway and does not play a detectable role in viral invasion (Deng et al., 2011; Gao et al., 2008; Liu & Howell, 2010; Liu et al., 2007; Nagashima et al., 2011; Zhang et al., 2015). The Turnip mosaic virus (TuMV) membrane-associated 6K2 protein is an inducer of bZIP60 splicing, which is necessary for the function of the UPR branch IRE1-bZIP60 in Arabidopsis (Zhang et al., 2015). It is known that several of these plant RNA viruses induce the UPR in order to benefit their replication and assembly (Yu et al., 2006; Zhang et al., 2015). The common feature shared by these plant virus-encoded UPR-inducing proteins is their association with lipid membrane or ER, and it is likely that membrane-associating proteins from other plant viruses will also be involved with ER

Fig. 2. PCD elicitation by TMV:p11 or PVX:p11 in systemic infected leaves. (a) PCD elicitation by wild-type p11 expressed from TMV (10 dpi). Necrotic symptoms are denoted by white arrowheads. (b) Diagram of p11 wild-type and transmembrane domain deletion (DTM) mutants. DTM1 and DTM2 are deleted for a single domain, DTM1A2 is deleted for both domains. (c) Stability of GarVX p11 wt and TM mutant PVX constructs during virus systemic movement was confirmed by RT-PCR with a p11 primer pair (Table S1). (d) PCD elicitation by wild-type and mutant p11 expressed from PVX (12 dpi). Necrotic symptoms are denoted by white arrowheads.
Garlic virus X (GarVX), a member of the genus Allexivirus (family Alphaflexiviridae) shares a similar genomic organization with carlaviruses, potexviruses and foveaviruses, and is widely detected in Allium species worldwide (Adams et al., 2004; Chen et al., 2004; Nam et al., 2015; Song et al., 1998; Wylie et al., 2011, 2014). Carlaviruses, potexviruses and foveaviruses have three genes, forming the triple gene block (TGB), that are involved in virus movement. Unusually, allexiviruses have only two of the three TGB genes. Previously, we demonstrated that TGBp2 (p11) encoded by ORF3 of GarVX was localized on plasmodesmata (PD), cytoplasm and ER in epidermal cells of Nicotiana benthamiana, and contributed to virus cell-to-cell movement (Lu et al., 2011). These experiments, p11 : RFP fusion formed granules with intense fluorescence on the ER. This direct association with the ER prompted us to investigate the ability of p11 to trigger the UPR in N. benthamiana.

To determine whether p11 triggers ER stress and induces the UPR, we firstly used Agrobacterium-mediated transient expression of p11 in N. benthamiana plants. The plant expression vectors were described previously (Lu et al., 2011). GarVX p11 fused to RFP (red fluorescent protein) or, as a control unfused RFP, each inserted into the binary vector pCV1300 (Lu et al., 2011), were transiently expressed in N. benthamiana via agroinfiltration (cell suspension OD600=1.0). Leaf samples were collected at 3 days post-infiltration (dpi) for analysis. Similarly, to examine expression from a virus, the p11 gene was cloned into an Agrobacterium-delivered PVX vector (Lacorte et al., 2010), and compared to a PVX : GFP control construct. Again, infiltrated leaf samples were collected at 3 dpi. Quantitative RT-PCR (qRT-PCR) was performed to evaluate the transcript levels of the UPR marker genes bZIP60 and BiP, using primer sets described previously (Ye et al., 2011). With both of these p11 expression systems, the transcription levels of BiP and bZIP60 were up-regulated (Fig. 1a). Binary plasmid-based p11 delivery resulted in 2- and 5.5-fold higher levels of bZIP60 and BiP, respectively, compared with RFP only or water-treated plants. During binary plasmid delivery, p11 : RFP expression was confirmed by confocal microscopy (Fig. 1b). Intracellular granules were observed as before (Lu et al., 2011), which were analogous to those seen in studies of PVX TGBp2 (Ju et al., 2005).

PVX infection was shown previously to induce the UPR (Ye et al., 2011, 2013). This was apparent in our experiments, where bZIP60 and BiP expression were each increased by approximately 2-fold when comparing control virus (PVX : GFP)-infected plants with uninoculated plants (Fig. 1a). However, bZIP60 and BiP were induced approximately 22.8- and 15.6-fold, respectively, in PVX : p11 infected patches compared to uninfected plants, and up-regulated approximately 10.6- and 9.5-fold, respectively, when comparing PVX : p11 expression to PVX : GFP infection (Fig. 1a). In this experiment, the level of PVX multiplication, as measured by coat protein accumulation, was the same for both constructs (Fig. 1c). We could not test whether p11 might boost UPR gene expression indirectly perhaps by raising the level or stability of the PVX TGBp3 protein (Aguilar et al., 2015). However, no RNA silencing suppression activity was detected for p11 in an infiltrated patch assay (Fig. S1, available in the online Supplementary Material).

To overcome this complication we decided to repeat these experiments but using Tobacco mosaic virus (TMV) to express p11, as a previous report showed that TMV infection does not affect the expression of UPR-related genes, including bZIP60 and BiP (Ye et al., 2013). When p11 was expressed from TMV in N. benthamiana, bZIP60 and BiP expression were increased by approximately 9-fold and 13.8-fold, respectively, when compared with plants infected with a control virus (TMV : GFP) (Fig. 1d). TMV infection by both viral constructs was confirmed by RT-PCR amplification of the TMV MP gene (Fig. 1e). These data clearly suggest that expression of GarVX p11 triggers the UPR in N. benthamiana by up-regulating expression of UPR-related genes.

The UPR has a linking role in the cellular response to biotic and abiotic stresses, influencing signaling pathways to either prolong cell survival or, under extreme stress, to initiate reactions leading to cell death (Williams et al., 2014). Thus, when the UPR fails to restore homeostasis following stress to the cell, apoptosis or PCD occurs (Martínez & Chrispeels, 2003; Urade, 2007). The PVX TGBp3, that up-regulates UPR-associated gene expression, was also found to elicit PCD when expressed in plants by TMV (Ye et al., 2013). In our study, strong necrotic symptoms were also observed on TMV : p11-infected systemic leaves (Fig. 2a), and similar necrotic symptoms occurred on systemic infected leaves when p11 was expressed from the PVX vector (Fig. 2d). These observations suggest that GarVX p11 could also elicit UPR-related cell death responses by over-expression of bZIP60 and BiP.

Our previous work identified two potential transmembrane domains (TM1 and TM2) in p11 (Lu et al., 2011). Thus, we constructed three p11 mutants to investigate whether membrane localisation, brought about by these TM domains, is linked to the p11-mediated UPR-related PCD (Fig. 2b). The TM1 and TM2 domains were deleted either individually or as a pair (Fig. 2b; mutants named as DTM1, DTM2 and DTM1A2). In the first experiments, the wild-type and mutant p11 genes were transiently expressed as GFP fusion proteins in leaves of the N. benthamiana 16c line, in which the ER network was revealed by transgenic expression of an ER-localizing form of GFP. Examination by confocal microscopy showed that all of the mutant and wild-type p11- mRFP proteins were expressed in the plants (Fig. 3a). Using free mRFP expressed from pCV : mRFP as a control, Western blotting was performed to confirm the intact expression of all the p11-mRFP fusion proteins (Fig. 3b). However, only the wild-type p11 fusion protein formed ER-
localized granules. Thus, both of the TM domains are required for granule formation and ER localization of p11 (Fig. 3a). When expressed from PVX, none of the TM mutants nor the control construct PVX : GFP elicited PCD in N. benthamiana, whereas the PVX : (wild-type) p11 construct did (Fig. 2d). In these experiments, the stability of the p11 gene in each of the PVX constructs was confirmed by RT-PCR (Fig. 2c). These results indicated that both of the p11 TM domains were necessary for the elicitation of UPR-related PCD and for the formation of p11 granules on the ER.

As well as responding to viral infection, bZIP60 expression is also induced during bacterial infection, and by treatment with heat or salt, or other chemicals such as tunicamycin (Tm) and dithiothreitol (DTT) (Deng et al., 2011; Liu et al., 2007; Martinez & Chrispeels, 2003; Moreno et al., 2012; Tateda et al., 2008). To further explore the mechanism of GarVX p11 activation of UPR-related PCD, we used the Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) system to knock down the expression of bZIP60 in N. benthamiana, as described previously (Ye et al., 2011).

Plants at the four leaf stage were infiltrated with mixed cultures of either Agrobacterium carrying TRV:00 (control virus), which was described previously (Jiang et al., 2014; Liu et al., 2002; Peng et al., 2011) or TRV : bZIP60 (silencing construct) to produce a systemic infection. Fourteen days after infection, qRT-PCR was performed to measure the expression of bZIP60. A 79% reduction in bZIP60 transcript level was detected in TRV : bZIP60-infected patches at 7 dpi Systemic leaves at 30 dpi

![Image](image_url)

**Fig. 3.** Knock-down of bZIP60 expression reduces p11-mediated necrosis. (a) Localization of wild-type and mutant mRFP-tagged p11 in transgenic line 16c plants expressing ER-GFP. mRFP is a control construct expressing unfused mRFP. Open triangles point to localization of wild-type p11 granules on ER. Bar, 25 µm. (b) Expression of wild-type and mutant mRFP-tagged p11 in transgenic line 16c plants at 2 dpi revealed by Western blotting of infiltrated plant extracts. Lower panel shows Ponceau staining of leaf proteins as loading control. (c) (i, ii, v, vi) Plants pre-infected with TRV:00; (iii, iv, vii, viii) plants pre-infected with TRV : bZIP60. Infiltrated leaves (i, ii, iii, iv) visualized at 7 days after PVX : p11 infection. Systemic leaves (v, vi, vii, viii) visualized at 30 days after PVX : p11 infection. White dashed circles indicate infiltrated patches. (d) qRT-PCR assay of bZIP60 transcript accumulation in leaves of TRV : 00 and TR : bZIP60 in N. benthamiana. qRT-PCR experiment followed the method described in the legend to Fig. 1. Bars represent the standard errors of the means of three replicates. ** denotes significant difference with \( P < 0.01 \). (e) Semi-quantitative RT-PCR assay was carried out which showed that GarVX p11 accumulation was at similar level in TRV : 00 and TRV : bZIP60 plants.
plants compared with TRV:00-infected plants (Fig. 3d). At the same time, the upper leaves of these plants were further infiltrated with PVX:p11. After a further 7 days, necrosis was observed in the PVX-p11-infiltrated patches, although, necrosis was significantly less severe in the TRV: bZIP60-silenced plants compared to the TRV:00-infected plants (Fig. 3c). After 30 days, the upper leaves systemically infected with PVX:p11 continued to exhibit much less necrosis in the TRV: bZIP60-treated plants compared to the TRV:00-treated plants. Semi-quantitative RT-PCR of RNA extracted from these leaves using p11-specific primers confirmed that the p11 insert was maintained in the PVX vector throughout this experiment and that the PVX:p11 virus accumulated to similar levels in both TRV:00- and TRV: bZIP60-infected plants (Fig. 3e). These results demonstrated that bZIP60, and therefore also the UPR pathway, is involved in the production of necrosis that is induced by expression of GarVX p11 from PVX.

Previous studies showed that inhibition of the IRE1-mediated UPR system, either by mutation of the IRE1A, IRE1B or bZIP60 genes or by use of VIGS to knockdown bZIP60 expression reduced the ability of PVX and TuMV to infect plants (Ye et al., 2011; Zhang et al., 2015). The mechanism of this effect is not yet known but it has been argued that high level expression of viral proteins and membrane alteration by formation of virus replication complexes might be toxic to cell survival in the absence of a functioning UPR system. Our experiments with the p11 protein suggest that successful infection of plants by GarVX is also likely to require interaction with UPR pathway components.

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